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Activity of Liposomal Amphotericin B against Experimental Cutaneous Leishmaniasis

VANESSA YARDLEY AND SIMON L. CROFT*

Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, United Kingdom

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The polyene antibiotic amphotericin B is currently a second-line treatment for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis. Lipid-amphotericin B formulations with lower toxicity than the parent drug that were developed for the treatment of systemic mycoses have proved to be an effective treatment for VL, especially AmBisome, a small unilamellar negatively charged liposome. In vitro, free amphotericin B was three to six times more active than the liposomal formulation AmBisome against both *Leishmania major* promastigotes in culture and amastigotes in murine macrophages. In a BALB/c *L. major* model of cutaneous infection, liposomal amphotericin B administered once a day on six alternate days by the intravenous route produced a dose-response effect between 6.25 and 50 mg/kg. Liposomal amphotericin B administered subcutaneously close to a lesion had no significant activity. Free drug was ineffective at nontoxic doses. The results suggest that liposomal amphotericin B may be useful in the treatment of cutaneous leishmaniasis.

The polyene antibiotic amphotericin B, a standard drug for the treatment of systemic fungal infections, is currently recommended as a second line of treatment for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL) (14, 40), especially when resistance to pentavalent antimonial drugs (18) and positivity for human immunodeficiency virus are factors (8). The use of amphotericin B is restricted by its acute toxicity and low therapeutic index and by the necessity for parenteral administration. Recently, several amphotericin B lipid formulations have been developed for the treatment of systemic mycoses (9, 36). These formulations have proved to have lower toxicity than free drug, mainly because their pharmacokinetic properties are different from those of free drug (17, 19). In general, large (>0.1-μm-diameter) lipid drug carriers are rapidly cleared from the circulatory system by the mononuclear phagocyte system, in particular the macrophages of the liver (Kupffer cells) and spleen. VL manifests itself in these cells, and so, serendipitously, drugs can be targeted to the site of an infection by *Leishmania donovani* parasites. Several amphotericin B-lipid formulations have now been tested against both experimental (15, 28, 37) and clinical (10, 11, 13, 34) infections caused by *L. donovani*, of which AmBisome (NeXStar Inc., San Dimas, Calif.), a unilamellar liposome, has been the most widely used (6, 7, 33). In experimental VL models, AmBisome accumulates in the liver, reaching therapeutic levels more quickly than conventional antimonial drugs (15). Due to its small size (<100 nm), AmBisome has been shown to stay in circulation longer than free drug (31) and subsequently to extravasate (36).

To date, the advances gained from drug delivery formulations have been applied primarily to the treatment of visceral disease, with little attention paid to cutaneous leishmaniasis (CL). Sodium stibogluconate-loaded liposomes were tested against established CL infections in mice (27) and demonstrated activity when administered intravenously but not subcutaneously. In other studies lipid-amphotericin B formulations showed little activity against experimental CL (29) and clinical MCL (22). This paper describes the activity of AmBisome against *Leishmania major* in vitro and a dose-response effect against an established infection in BALB/c mice.

* Corresponding author. Mailing address: Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel St., London WC1E 7HT, United Kingdom. Phone: (44) 171 927 2345. Fax: (44) 171 636 8739. E-mail: s.croft@lshtm.ac.uk.

MATERIALS AND METHODS

Drugs and formulation. AmBisome and liposomes without drug were kindly provided by R. Proffitt (NeXStar Inc.). Each vial of AmBisome contained 50 mg of amphotericin B as lyophilized phospholipid vesicles which were reconstituted by the addition of cold, sterile distilled water to obtain a stock solution of 4 mg of amphotericin B/ml and, after warming, diluted to the required concentration with cold, sterile 5% dextrose for in vitro and in vivo use. Unloaded liposomes were administered at a lipid concentration equivalent to 50 mg of AmBisome/kg of body weight. Each vial of Fungizone (E. R. Squibb & Son Ltd., Hounslow, United Kingdom), both in tissue culture and in intravenous formulations, contained 50 mg of amphotericin B in association with sodium desoxycholate and sodium phosphate buffer, which was prepared as a stock solution of 5 mg of amphotericin B/ml with 5% dextrose. Pentostam (100 mg of Sb^V/ml) and powdered sodium stibogluconate ($NaSb^V$) were kindly provided by Glaxo-Wellcome, Dartford, United Kingdom. For the in vitro assays Pentostam was diluted with medium to the required concentration. Sodium stibogluconate powder was formulated in 0.25% methylcellulose prior to in vivo administration.

Parasites. Two strains of parasite were used in the study: *L. major* MRHOM/SU/59/NEAL-P and *L. major* MHOM/SA/85/JISH118. Promastigotes of each strain were maintained in Schneider's medium (Gibco, Paisley, United Kingdom) with 10% heat-inactivated fetal calf serum (Harlan Sera-Lab., Crawley, United Kingdom) at 24°C.

TABLE 1. In vitro activity of amphotericin B and liposomal amphotericin B (AmBisome) against *L. major* promastigotes

Treatment	ED_{50} (μg/ml) (95% confidence limits) for *L. major* strain[a]:	
	NEAL-P	JISH118
$NaSb^{Vb}$	≥100	>100
Amphotericin B	0.5 (0.1–1.7)	0.96 (0.21–1.7) (A)
Liposomal amphotericin B	2.96 (1.2–3.7)	3.21 (2.0–4.9) (A)
Liposomes without drug	—[c]	—

[a] Values followed by the same letter are significantly different (NEAL-P, $0.05 < P < 0.1$; JISH118, $0.01 < P < 0.02$). There was no significant difference in activity between strains ($P > 0.05$).
[b] Values are for Sb^V only.
[c] —, inactive.

TABLE 2. In vitro activity of amphotericin B and liposomal amphotericin B against *L. major* amastigotes in macrophages

	ED_{50} (μg/ml) (95% confidence limits) for *L. major* strain[a]:	
	NEAL-P	JISH118
$NaSb^{V}$[b]	30 (29.7–41.3)	35 (28.7–40.3)
Amphotericin B	0.2 (0.11–0.82) (A)	0.6 (0.3–0.95) (B)
Liposomal amphotericin B	1.8 (0.98–2.6) (A)	2.0 (1.1–5.2) (B)
Liposomes, without drug	—[c]	—

[a] Significance: A, $P < 0.05$; B, $P < 0.02$.
[b] Values are for Sb^{V} only.
[c] —, inactive.

In vitro assays. Promastigotes (10^6/ml) were incubated in the presence of drug for 48 h. MICs and 50% effective doses (ED_{50}s) for each strain were determined microscopically with a Neubauer hemocytometer.

CD1 mouse (Charles River Ltd., Margate, United Kingdom) peritoneal macrophages were harvested 24 h after starch (Sigma) induction and dispensed into eight-well Lab-tek (Nunc Inc., Naperville, Ill.) tissue culture slides at a concentration of 5×10^4/well. After 24 h the cells were infected with *L. major* NEAL-P or JISH118 amastigotes harvested from lesions of infected mice at a ratio of five parasites to one macrophage. After a further 24 h the infected cells were exposed to drug for 5 days, with fresh drug in the cell overlay being replaced on day 3. The experiment was terminated by methanol fixation of the slides, followed by Giemsa staining. The slides were examined microscopically to count the percentage of cells infected. ED_{50}s were determined by linear regression analysis, 95% confidence limits were calculated, and *P* values were calculated with the Student *t* test.

In vivo assays. Eight- to ten-week-old, female BALB/c mice (Tuck & Son Ltd., Hullbridge, United Kingdom) were infected with 10^7 *L. major* JISH118 or NEAL-P first-passage, stationary-phase promastigotes subcutaneously at the base of the tail. At 3 to 4 weeks postinfection, lesions were measured with calipers in two dimensions, mean diameters were determined, and mice were randomly sorted into groups of five prior to dosing. In the first series of experiments groups of mice were given 0.25% methyl cellulose subcutaneously (s.c.) once a day for 10 days; sodium stibogluconate (400 mg of Sb^{V}/kg) s.c. once a day for 10 days; or Fungizone, AmBisome, or liposomes without drug administered either s.c. (close to the lesion) or intravenously (i.v.) once a day on six alternate days. The amphotericin B preparations were given on alternate days to avoid macrophage blockade and optimize activity (11a). The lesions were measured weekly, and the percent increase or decrease in size was calculated. In a second series of experiments mice with established lesions were treated with a range of doses, administered six times (once a day on alternate days) of liposomal amphotericin B by the i.v. route only; control groups received single-dose levels of sodium stibogluconate (s.c.), methylcellulose (s.c.), or amphotericin B (i.v.).

All experiments were conducted, under license, according to United Kingdom Home Office regulations.

RESULTS

In vitro amphotericin B was four- to sixfold more active against *L. major* promastigotes than AmBisome (Table 1). This difference between the two formulations was significant for the JISH118 strain ($P < 0.02$). Sodium stibogluconate, as previously reported, was inactive against promastigotes. Amphotericin B was four- to ninefold more active than AmBisome against intracellular *L. major* amastigotes in macrophages (Table 2). The difference in activity of the formulations was significant for both strains ($P < 0.05$ and $P < 0.02$ for NEAL-P and JISH118, respectively), and they were 50- to 100-fold more active than sodium stibogluconate; *L. major* amastigotes are less sensitive to pentavalent antimonials than *L. donovani* (2a). There was no significant difference in the in vitro sensitivity of the two strains of *L. major* to any of the formulations tested ($P > 0.5$).

In the first in vivo study the activities of amphotericin B and

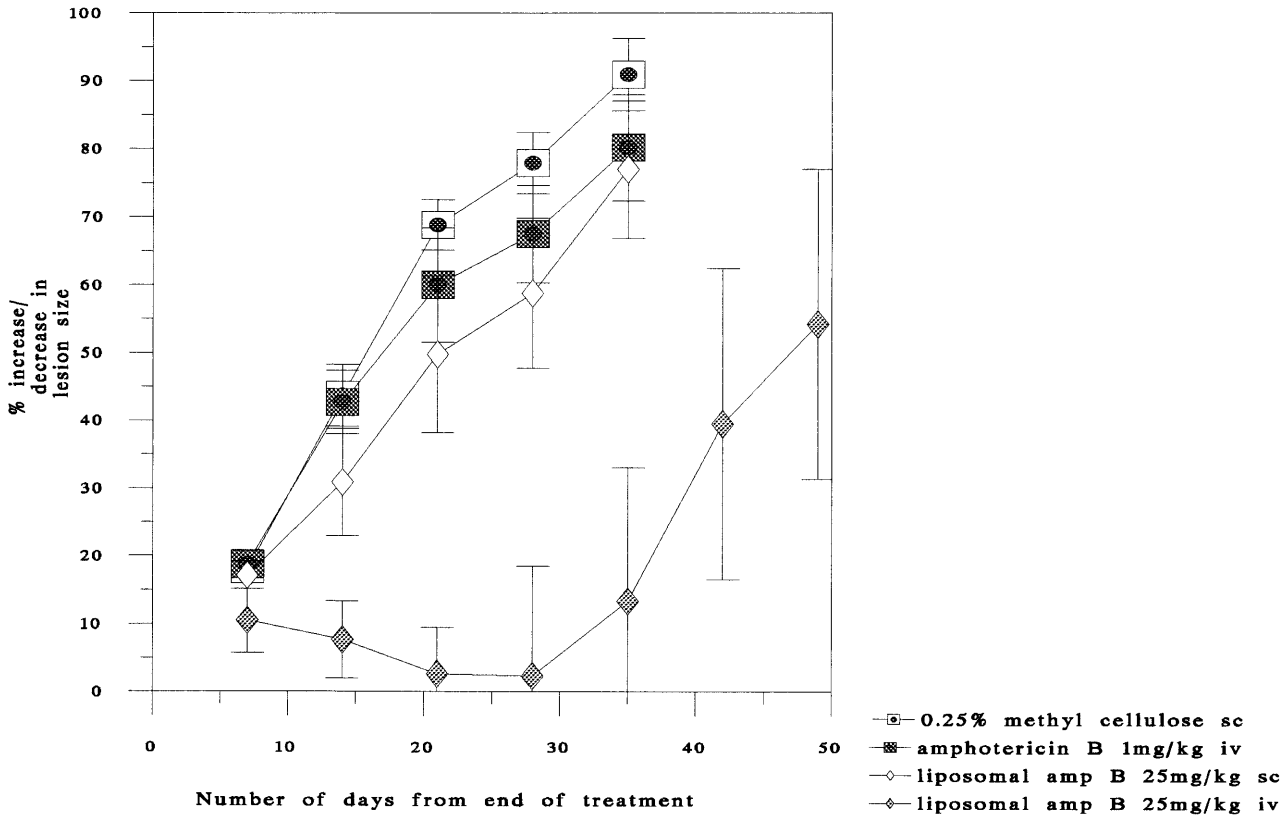

FIG. 1. Comparison of i.v. and s.c. (close to the lesion) administration of liposomal amphotericin B (amp B) in BALB/c mice infected with *L. major* JISH118. Values are means ± standard errors of the means.

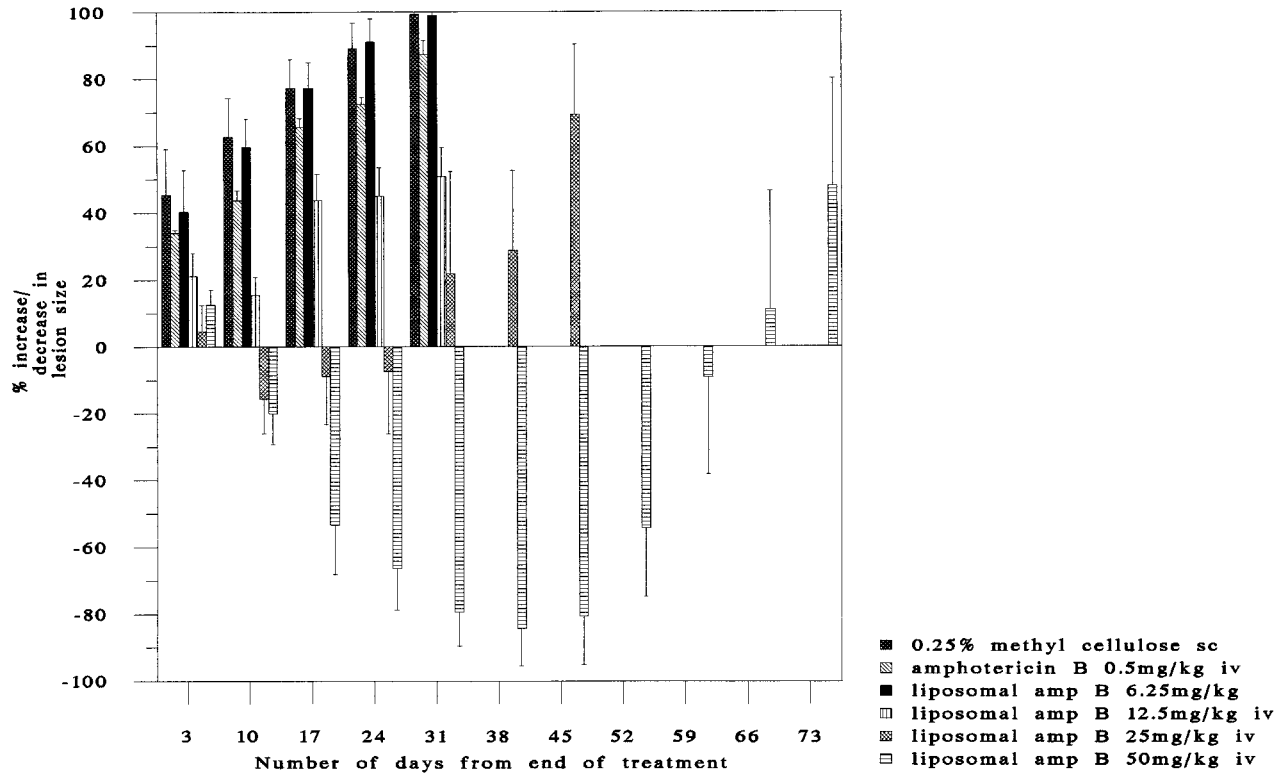

FIG. 2. Activity of liposomal amphotericin B (amp B) against *L. major* NEAL-P in BALB/c mice. Values are mean percent increase or decrease ± standard errors of the means.

liposomal amphotericin B, administered i.v. and s.c., against *L. major* were compared. Mice treated with AmBisome at a dose of 25 mg/kg showed significant reduction in lesion size in comparison to mice treated with methylcellulose (negative control) (Fig. 1); for example, 2 weeks from completion of treatment, *P* was between 0.01 and 0.02. In two of the five mice, lesions completely resolved, although they recurred after completion of treatment. Activity was also seen in mice treated with liposomal amphotericin B at 12.5 but not at 6.25 mg/kg i.v. (data not shown). There was a small, insignificant reduction in lesion size in these mice compared to controls when 25 mg of AmBisome per kg was injected s.c. close to the lesion. Fungizone at 1 mg/kg, administered i.v. or s.c. close to the site of the lesion, was inactive (Fig. 1). Doses did not exceed 1 mg of free amphotericin B/kg, as above this level acute toxicity was observed. The standard drug sodium stibogluconate had little effect in this model, even at a dose of 400 mg of Sb^V/kg administered s.c. daily for 10 consecutive days.

In a second in vivo experiment, involving the two strains of *L. major*, NEAL-P and JISH118, AmBisome administered by the i.v. route at doses between 50 and 6.25 mg/kg showed a dose-response effect (Fig. 2 and 3). At 50 mg/kg the lesions of four of five mice resolved completely between 4 and 6 weeks following the completion of treatment. However, none of the mice had a parasitological cure, as lesions redeveloped in all mice. At 25 and 12.5 mg/kg, liposomal amphotericin B also caused lesions to diminish. This was probably due to a reduction in the rate of lesion development rather than to a reduction in lesion size (Fig. 2 and 3). Lesion size was monitored for 7 weeks after termination of treatment, by which time all cured mice had relapsed; animals were removed from the experiment as soon as no effect or distress was observed, usually 5 weeks after the end of treatment. The studies confirmed that a high dose (400 mg of Sb^V/kg) of sodium stibogluconate and 0.5 mg of free amphotericin B per kg caused no reduction in size of the lesions of either strain of *L. major* (Fig. 2 and 3). A lower dose of Fungizone was used in this experiment to reduce the acute toxicity observed in the previous experiment.

DISCUSSION

Notable differences were observed between the in vitro and in vivo activities of the liposomal amphotericin B formulation (AmBisome) against *L. major* parasites. In vitro, free amphotericin B was more than four times more active against both extracellular promastigotes and intracellular amastigotes in mouse peritoneal macrophages than the liposomal amphotericin B formulation. Possible explanations for these in vitro results relate to uptake and availability of the drug. With regard to promastigotes, free amphotericin B, although it is highly protein bound, is able to associate directly with the parasite membrane ergosterol, for which it has a high affinity (4, 32), whereas amphotericin B in the liposomal formulation is associated with both distearoylphosphatidyl glycerol and cholesterol within the synthetic phospholipid bilayer and is probably less available. The presence of free amphotericin B bound to protein in the medium may improve drug activity in the amastigote-macrophage model, where uptake is by endocytosis following the interaction of serum components such as high- and low-density lipoproteins (17, 39). Lipid-intercalated amphotericin B can also bind to serum components; the degree of affinity of this association depends upon the nature of the

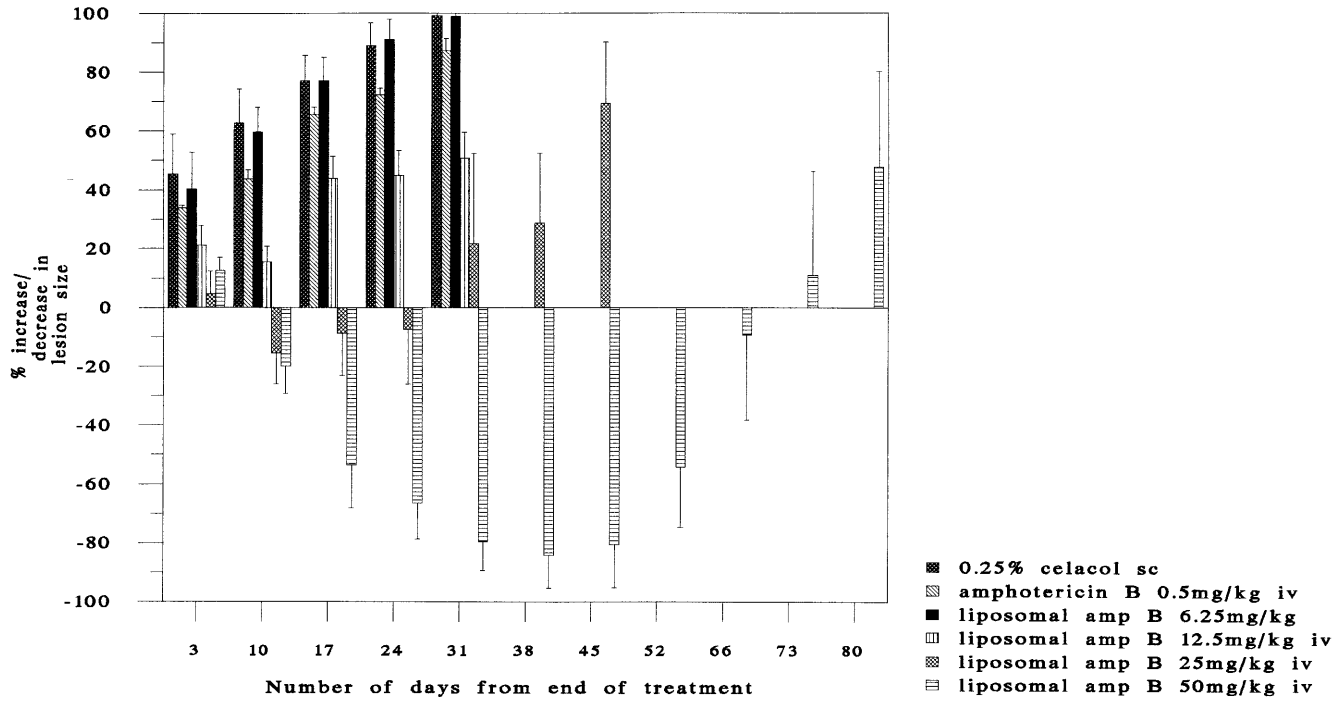

FIG. 3. Activity of liposomal amphotericin B (amp B) against *L. major* JISH118 in BALB/c mice. Values are mean percent increase or decrease ± standard errors of the means.

carrier and the ease with which it can be disrupted by these components (38). However, AmBisome is a rigid liposome formulation (i.e., the bilayer is in the gel phase at the physiological temperature), which is not easily disrupted by low- and high-density lipoprotein (38), and it could thus be hypothesized that AmBisome enters the macrophage by phagocytosis and is transported to the parasitophorous vacuole via the endosomal pathway (30) rather than by interaction with serum components. It has been shown that the accumulation of liposomal amphotericin B, specifically with AmBisome, by the J774 macrophage cell line is 10-fold lower than that of free drug (21), and these results indicate this also.

Lipid-amphotericin B formulations are a successful treatment for VL, as the site of infection is an ideal target for passive drug delivery systems. However, in CL caused by *L. major* the main site of infection is within the dermis (5, 12), which is a more difficult target for drug delivery systems. Previously it was shown that liposomal sodium stibogluconate is more efficacious than free drug against experimental CL and that the route of administration is an important factor in the effectiveness of the drug (27). However, studies with other lipid-amphotericin B formulations have reported little or no effect against experimental CL (29) or clinical MCL (22). One report described AmBisome as effective against antimony-resistant clinical CL, but liposomal dosage was followed by several weeks' treatment with amphotericin B (35). Our studies have confirmed that parenterally administrated AmBisome reduces lesion size in experimental CL infections in a dose-dependent manner but that the route of administration is important: i.v. is better than s.c. administration. A few weeks after treatment the lesions recurred, indicating that liposomal amphotericin B had a suppressive rather than a curative effect in this model. Since the BALB/c mouse is highly susceptible to *L. major* due to the Th2 cell-associated response particular to this parasite in this strain (23), with development of a disseminating "noncure" infection any amelioration of infection is significant. Even "self-cure" mouse strains infected with CL sequester parasites (25, 26), and infection will cause relapse if the mouse is subsequently immunosuppressed (1).

The pharmacokinetics and activity of AmBisome in the therapy of CL remain to be elucidated. AmBisome, by being small, stable, and negatively charged, has reduced interaction with the biological milieu and remains in the circulatory system longer than free amphotericin B (31, 38). At the site of infection in CL, the blood vessels are leaky due to local inflammatory responses (24), possibly resulting in extravasation of liposomes at the site of infection. This effect has been described with other liposome formulations in relation to dermal pathologies (20, 41). It has been suggested that although liposomes reach the dermis, drug accumulation is insufficient to reach a curative level (16). However, our study proves that therapeutic levels can be achieved following intravenous administration. Intralesional administration of AmBisome had no suppressive effect, probably due to rapid removal of the liposomes by the draining lymph nodes proximal to the lesion and the subsequent reduction in circulating amounts due to first passage through the liver (2). These experiments demonstrate the possible use of intravenous AmBisome against CL, albeit at higher doses than those required for visceral disease. In the visceral model of leishmaniasis a single dose of liposomal amphotericin B as low as 1 mg/kg is able to clear more than 50% of the parasites (6).

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